CCL17. These results suggest that M2 macrophages stimulated by RANKL/RANK signaling might recruit Tregs into the tumor microenvironment of EMPD, which thereby induces an immunosuppressive microenvironment in EMPD.

Gordon and Martinez (2010) classified the development of monocytes into mature and fully activated macrophages into three successive stages. During the third phase of activation, macrophages reach a mature functional phenotype in response to microbial and opsonic stimuli such as antibody complexes. In the present study, macrophages induced by M-CSF and IL-4 did not produce a substantial amount of CCL17; however, when these macrophages were stimulated by sRANKL, they produced CCL17 in an sRANKL dose-dependent manner. These results suggest that sRANKL may be another stimulus during the third phase of macrophage activation.

Denosumab, a fully human monoclonal antibody for RANKL, is used clinically to treat metastatic bone tumors (Azim and Azim, 2013). Our present study suggests that RANKL targeting with denosumab can be used in conjunction with the therapeutic elimination of primary EMPD to prevent local immunosuppression and metastatic disease.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Cathelicidin LL-37 Induces Semaphorin 3A Expression in Human Epidermal Keratinocytes: Implications for Possible Application to Pruritus

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TO THE EDITOR

Higher density of epidermal nerve fibers has been associated with itch sensitization in the periphery, with nerve fiber density being higher in atopic dermatitis (AD) lesional skin than in normal skin (Tominaga and Takamori, 2014). Concomitantly, the epidermal levels of the nerve repulsion factor semaphorin 3A (Sema3A) are lower in AD patients and AD model NC/Nga mice than in controls (Tominaga *et al.*, 2008). Sema3A replacement in AD model NC/Nga mice normalized hyperinnervation, resulting in the suppression of itching (Yamaguchi *et al.*, 2008; Negi *et al.*, 2012).

To defend against pathogens, human skin contains antimicrobial peptides, including cathelicidins and β-defensins, which are induced by inflammation (Schauber and Gallo, 2008; Niyonsaba *et al.*, 2009). The induction of these peptides, such as cathelicidin LL-37 and human β-defensins, produced by epidermal keratinocytes is impaired in lesional skin in patients with AD, explaining their frequent infections (Ong *et al.*, 2002; de Jongh *et al.*,

Abbreviations: AD, atopic dermatitis; NHEK, normal human epidermal keratinocyte; $P2X_7R$, $P2X_7$ receptor; Sema3A, semaphorin 3A

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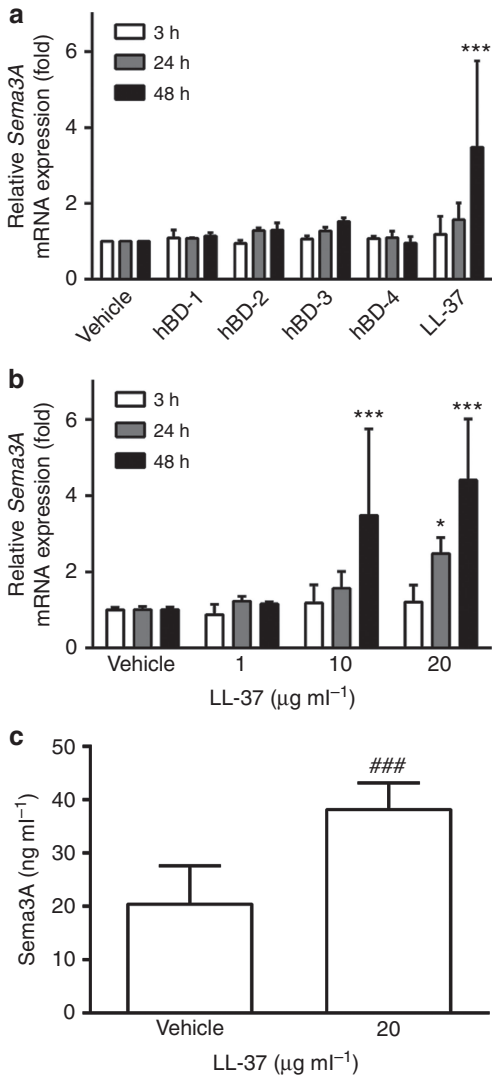

Figure 1. Effects of antimicrobial peptides on Sema3A expression in cultured NHEKs. (**a**) NHEKs were incubated with 10 μg ml^{-1} hBD-1-4, LL-37, or medium alone (vehicle) for 3, 24, or 48 hours, and *Sema3A* mRNA expression was assayed by quantitative real-time PCR. *Sema3A* mRNA levels were normalized relative to those of *RPS18*. (**b**) Effects of LL-37 concentration on *Sema3A* mRNA expression. Values represent fold changes compared with vehicle. LL-37 induced *Sema3A* expression dose- and time dependently. (**c**) NHEKs were incubated with LL-37 for 48 hours, and the concentrations of Sema3A in the cell-free supernatants were determined by ELISA. $^{*}P<0.05$, $^{***}P<0.0001$ (two-way ANOVA followed by Dunnett's test), $^{\#\#\#}P<0.0001$ (two-tailed Student's *t*-test) compared with vehicle. All values represent the mean ± SD of 3–6 experiments. ANOVA, analysis of variance; hBD, human β defensin; NHEK, normal human epidermal keratinocyte; RPS18, ribosome protein S18; Sema3A, semaphorin 3A.

2005). However, the interrelationships among Sema3A and antimicrobial peptides are unclear. We therefore investigated the effects of antimicrobial peptides on Sema3A expression in cultured normal human epidermal keratinocytes (NHEKs), as well as the signaling pathways involved in LL-37-induced *Sema3A* expression.

NHEKs derived from adult epidermis (Lonza, Basel, Switzerland) were cultured in KBM-Gold (Lonza), a serum-free medium containing a low concentration (0.15 mM) of calcium, at 37 °C with 5% CO_2 and used within three passages. LL-37 (L^{1}LGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES^{37}) was synthesized by the solid phase method on a peptide synthesizer (model PSSM-8; Shimadzu, Kyoto, Japan). NHEKs were incubated with human β-defensin-1–4 (Peptide Institute, Osaka, Japan) or LL-37, and the levels of *Sema3A* mRNA expression at different time points were assessed by quantitative real-time PCR using SYBR Premix Ex Taq (TaKaRa, Kyoto, Japan) and the primers 5′-ACCCAACTATCAATGGGTG CCTTA-3′ (forward) and 5′-AACACTG GATTGTACATGGCTGGA-3′ (reverse). As controls, *ribosome protein S18* mRNA was amplified using the primers 5′-TTTGCGAGTACTCAACACCAACATC-3′ (forward) and 5′-GAGCATATCTTCGGC CCACAC-3′ (reverse).

Incubation with LL-37 for 48 hours increased *Sema3A* expression, whereas the individual human β-defensins had little effect (Figure 1a). This LL-37-induced *Sema3A* expression was dose- and time dependent (Figure 1b). Furthermore, Sema3A protein levels measured using ELISA kits (USCN Life Science, Wuhan, China) were markedly higher in the supernatants of NHEKs incubated with LL-37 than in control supernatants (Figure 1c).

LL-37 has been shown to activate rat mast cells via G protein–coupled receptors (Niyonsaba *et al.*, 2001). Therefore, the effects of cholera toxin (Wako Pure Chemical Industries, Ltd. Osaka, Japan) and pertussis toxin (Sigma-Aldrich, St Louis, MO), inhibitors of the G_s and G_i subfamilies of G protein α-subunit, respectively, on *Sema3A* induction in cultured NHEKs were analyzed. LL-37-induced *Sema3A* expression was completely inhibited by pretreatment with pertussis toxin but not cholera toxin (Figure 2a).

Activation of either mitogen-activated protein kinase or phosphatidylinositol 3 kinase was found to be involved in the LL-37 signaling pathway (Niyonsaba *et al.*, 2010). We therefore assessed the effects of mitogen-activated protein kinase and phosphatidylinositol 3 kinase inhibitors on *Sema3A* induction in cultured NHEKs. LL-37-induced *Sema3A* expression was inhibited by pretreatment with the extracellular signal-regulated kinase 1/2 inhibitor PD98059 (Cell Signaling Technology, Beverly, MA), but not the phosphatidylinositol 3 kinase inhibitor wortmannin (Sigma-Aldrich), the p38 inhibitor SB203580 (Sigma-Aldrich), and the c-Jun N-terminal kinase inhibitor SP600125 (Calbiochem, Darmstadt, Germany; Figure 2b).

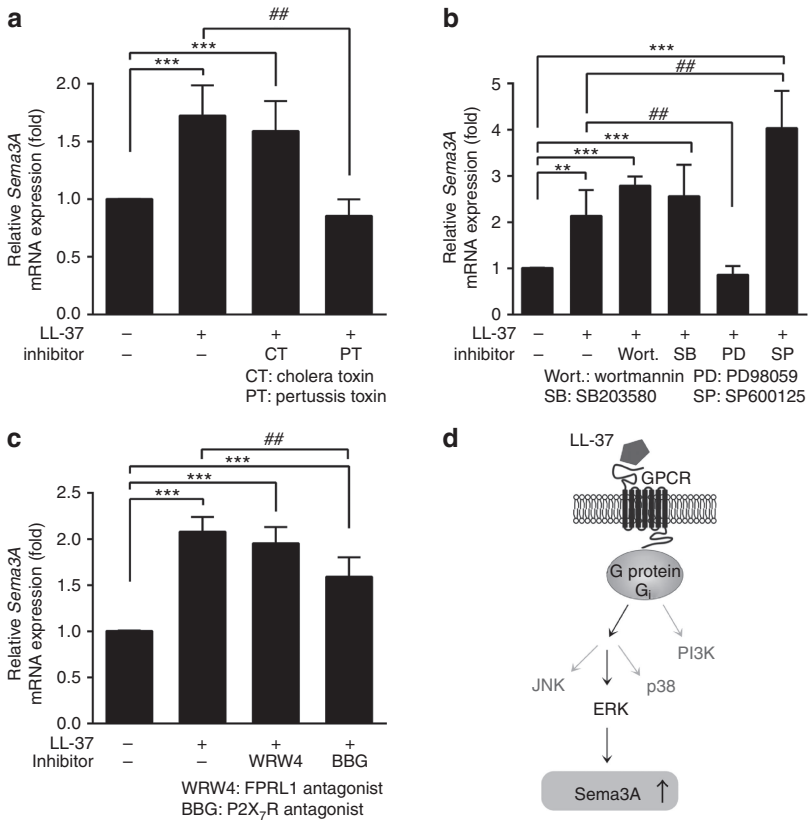

Figure 2. Analysis of the signaling pathway involved in LL-37-induced *Sema3A* expression. NHEKs were pretreated with 100 μg ml^{-1} cholera toxin (CT) (**a**), 200 ng ml^{-1} pertussis toxin (PT) (**a**), 20 μM wortmannin (Wort.) (**b**), 10 μM SB203580 (SB) (**b**), 10 μM PD98059 (PD) (**b**), 10 μM SP600125 (SP) (**b**), 1 μM WRW4 (**c**), 1 μM BBG (**c**), or vehicle (−) for 1 hour, followed by incubation for 24 hours with 20 μg ml^{-1} LL-37, and *Sema3A* expression was assayed by quantitative real-time PCR. Values represent fold changes compared with vehicle and the mean ± SD of 4–6 experiments. $^{**}P<0.01$ and $^{***}P<0.001$ compared with vehicle, $^{\#\#}P<0.01$ compared with LL-37 alone (one-way ANOVA followed by Dunnett's test). (**d**) Schematic diagram of the signaling pathway of LL-37-induced *Sema3A* expression in NHEKs. ANOVA, analysis of variance; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NHEK, normal human epidermal keratinocyte; PI3K, phosphatidylinositol 3 kinase; Sema3A, semaphorin 3A.

We next examined whether formyl peptide receptor-like 1 and $P2X_7$ receptor ($P2X_7R$), both candidate LL-37 receptors (Niyonsaba *et al.*, 2009), are involved in *Sema3A* induction in cultured NHEKs. LL-37-induced *Sema3A* expression was partially inhibited by pretreatment with the $P2X_7R$ antagonist BBG (Tokyo Chemical Industry, Tokyo, Japan) but not by pretreatment with the formyl peptide receptor-like 1 antagonist WRW4 (ABGENT, San Diego, CA; Figure 2c).

Taken together, these findings showed that the antimicrobial peptide LL-37 markedly upregulated the expression of Sema3A mRNA and protein in cultured human keratinocytes, whereas the four human β-defensins were inactive (Figure 1), indicating that LL-37 is an endogenous inducer of Sema3A expression in keratinocytes. Vitamin D3, which increases cathelicidin expression in keratinocytes (Schauber and Gallo, 2008), might therefore also drive the LL-37-Sema3A pathway.

We found that certain G_i-coupled receptors are involved in *Sema3A* induction (Figure 2a). Although LL-37 has been shown to activate G protein–coupled receptors, such as formyl peptide receptor-like 1, $P2X_7R$, and Mas-related G protein–coupled receptor X2 (MrgX2; Barlow *et al.*, 2014), LL-37 receptors are less well-characterized in human keratinocytes. We showed that LL-37-induced *Sema3A* expression was partly inhibited by a $P2X_7R$ antagonist, but not by a formyl peptide receptor-like 1 antagonist (Figure 2c), and that *MrgX2* mRNA expression was undetectable in NHEKs (Umehara *et al.*, unpublished observation). These findings suggest that, although other receptors may be involved in induction, LL-37 activation of $P2X_7R$ is at least partly involved in *Sema3A* induction in keratinocytes. We also found that LL-37-induced *Sema3A* expression was completely inhibited by PD98059 (Figure 2b), indicating that extracellular signal-regulated kinase 1/2 signaling may be required for *Sema3A* induction. In contrast, SP600125 increased rather than inhibiting *Sema3A* expression (Figure 2b), suggesting that c-Jun N-terminal kinase signaling is involved in the suppression of *Sema3A* expression in keratinocytes. Altered c-Jun N-terminal kinase signaling might reduce Sema3A production in AD. Taken together, these findings suggest that LL-37 may bind to certain G_i-

coupled receptors, including $P2X_7R$, activating the extracellular signal-regulated kinase 1/2 signaling pathway, and finally inducing Sema3A expression in human epidermal keratinocytes (Figure 2d).

Although 70–90% of patients with psoriasis also have pruritus, they are rarely accompanied by intense itch as in AD (Yosipovitch *et al.*, 2000; Reich and Szepietowski, 2007). We previously showed that Sema3A production was not reduced in the epidermis of psoriatic patients with or without itch (Taneda *et al.*, 2011). In contrast to AD, high levels of expression of several antimicrobial peptides including LL-37 were observed in psoriatic lesions (Ong *et al.*, 2002; Nomura *et al.*, 2003), suggesting that epidermal Sema3A expression is not reduced in these patients. These findings suggest that LL-37 may restore Sema3A production in keratinocytes of certain pathological conditions such as psoriasis and that the decreased epidermal Sema3A production in AD lesional skin may be partly caused by a breakdown in this recovery system. Thus, the topical application of LL-37, which not only has antimicrobial activity but also enhances Sema3A production, might be a useful therapeutic strategy for AD.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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IL-10 Signaling in Dendritic Cells Attenuates Anti-*Leishmania major* Immunity without Affecting Protective Memory Responses

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TO THE EDITOR

Leishmania major (*L. major*) parasites cause skin lesions and are transmitted by sand flies infecting millions of people each year. Upon infection, the parasites (promastigotes) are phagocytosed by macrophages where they transform into amastigotes and replicate. The amastigotes are eventually released and taken up by dendritic cells (DCs), which activate naive T cells in skin-draining lymph nodes (sdLNs). In *L. major*–infected humans and mice on a resistant background, DCs induce an IFNγ-driven T-helper (Th) type-1/T cytotoxic (Tc) 1 response that stimulates

Abbreviations: DC, dendritic cell; IFN, interferon; IL, interleukin; L. major, Leishmania major; sdLN, skin-draining lymph node; Th, T helper; Treg, regulatory T cell

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